IPM program with emphasis in Biological Control with a highly pathogenic *Beauveria bassiana* mixed strains as a strategy to overcome insecticide resistance in the most important insect-pest of coffee crops: the Coffee Berry Borer

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INTRODUCTION

Coffee is cultivated by some 20 millions farmers in more than 50 countries in Africa, Asia and America, and generates an industry that surpasses USD 70 billion annually. Coffee cultivars are planted in a very wide range of ecological and social conditions and may be grown using few culture practices where almost no changes occur to the natural environment or by means of mechanization, irrigation, fertilization and pest control with chemical insecticides which often leads to a complete destruction of the surrounding vegetation and changes in the ecosystem. Small farmers are the predominant coffee growers in all countries and technology in most cases is not readily available to them. This coffee world situation results in a great variety of sanitary problems, which varies according to the country even in similar ecological regions. All agricultural production systems have to deal with plant protection problems and coffee is no exception. Since the early 20th century, coffee production suffers from numerous insect pests, being the coffee berry borer, the white stem borer, leaf miners and mealybugs, the most serious examples since they can cause coffee farmers to lose up to 20% of a crop and reduce the coffee value by 30 to 40%. These coffee pests could be kept below economic threshold levels by adopting integrated management strategies such as anticipation and continuous monitoring of pest outbreaks, maintenance of optimum shade, pruning of coffee bushes, good harvesting and processing of the berries, conservation and augmentation of indigenous natural enemies, introduction of exotic natural enemies and timely use of need based chemical or bio insecticides. This chapter deals with the most important insect pest affecting coffee plantations in all coffee producing countries, the Coffee Berry Borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae: Scolytinae), a brief description of an integrated pest management program that can help reducing the insect populations, the appearance of resistant insects in the field after spraying inadequately endosulfan for several years and a description of a novel strategy to select a highly pathogenic mixed strains of fungi in order to overcome this resistance and maintain infestation in the field under threshold levels.

Life cycle and damage caused by *Hypothenemus hampei*

The life cycle of Coffee Berry Borer has been studied by several authors (Corbett 1933; Bergamin 1943; Ticheler 1963; Baker 1984; Muñoz 1989; Decazy 1990a; Baker *et al.* 1992; Jaramillo *et al.* 2009), under different temperature and ambient conditions. In Colombia several studies have been carried out under laboratory and field conditions (Montoya y Cárdenas 1994; Gaviria *et al.* 1995; Ruíz *et al*. 1996; Ruíz 1996). The adult female coffee berry borer is a small black beetle, 1.5 mm in length, longer and slightly wider than the male. Its entire immature life stages are spent inside the coffee berry. Males mate inside the berry with females and never emerge. Males live between 50 and 75 days, while females from 100 to 150 days. The female beetle enters the coffee berry and borers a tunnel inside the coffee bean where lays eggs at a rate of 2-3 per day up to 20 days. The average number of offspring produced per female is 74 and the total life cycle was calculated to be 27.5 days (24.5 °C), however, a complete development of coffee berry borer from egg to adult in Colombia is estimated between 45 to 60 days under conditions of 21ºC and 19ºC, respectively (Ruiz 1996, Bustillo 2002). The life cycle in degree – days is 237.2 with a threshold temperature development of 16.5 ºC. Even though there are reports of non- mated females giving origin to fertile eggs (Montoya y Cárdenas 1994, Muñoz 1989, Barrera *et al.* 1995), this has not being verified experimentally (Alvarez y Cortina 2004). Mated

females emerge to fly and look for a new berry. Host finding is achieved initially by responding to volatiles emitted by the coffee berry during its development and close orientation to the berries may be assisted by vision preferring the red berries (Bustillo *et al*. 1998).

Hypothenemus hampei has a reproductive behavior that ensures a high degree of inbreeding. Female-biased sexual ratio was estimated to be 1:10 males to females (Bergamin 1943). Females mate with their flightless male siblings when still inside the berry, so they leave the infested berry already fertilized. Although cytological examination of somatic cells in males proved that they were diploid, males failed to express paternally derived alleles and then transmitted only their maternally derived chromosomes; thus, *H. hampei* is functionally haplo-diploid (Brun *et al*. 1995). It has been suggested that the unusual sex determination and skewed sex ratios favoring females is caused by the infection of *Wolbachia* in *H. hampei* (Vega *et al*. 2002, Benavides 2005); However, the presence of males in the coffee berry borer populations can be explained by the presence of an extra chromosome in male cells (Bergamin and Kerr 1951).

The damage caused by *H. hampei* is mainly a decrease of coffee yield due to abscission of berries, loss of weight, and a decrease on coffee quality and, therefore, coffee price. It has been estimated that there is a weight loss of 55% on coffee grains attacked by *H. hampei* (Montoya 1999); however, the decrease of weight of the total coffee production is about 18% Borbón 1990). *Hypothenemus hampei* also attacks young berries in formation (less than 20 weeks after flowering) which results in the abscission of 32% of coffee berries (Mendoza 1996). Furthermore, *H. hampei* causes yield losses as high as 40-80% at a field infestation of 90% (Le Pelley 1968). Coffee prices are greatly reduced when the beans exhibit *H. hampei* damage. International marketing policies do not allow coffee for exportation that have more than 1.5% damage caused by insects. Thus, the price of coffee in producing countries is severely reduced if the levels of infestation with *H. hampei* are greater (Duque and Baker 2003).

Distribution and dispersion of *Hypothenemus hampei*

The coffee berry borer dispersal through the world has been documented (Benavides 2005; Benavides *et al.* 2005), using molecular tools based on AFLP. Results suggested that invasion in Asia and America came from West Africa. The distribution of the fingerprints and its genetic relationship determined by a neighbor - joining analysis, showed that there were two introductions of Coffee Berry Borer in Brazil, then dispersed into American countries and a third introduction was evident in Peru and Colombia (Benavides 2005).

Hypothenemus hampei has now invaded all coffee producing countries worldwide (Table 1). It was first detected in Colombia in 1988 and is found now in all Colombian coffee plantations infesting near 800.000 ha and affecting the assets of more than a half million of Colombian coffee producing families. The coffee cultivars in Colombia have been kept free of important insect pests since the beginning of its development as a commercial exploitation. Only a few records exists on the attack of minor pests such as: *Leucoptera coffeellum* (Guerin - Méneville), *Coccus viridis* (Green), *Planococcus citri* (Risso), *Dysmicoccus brevipes* (Cockerell), *Puto barberi* (Murillo), and the red spider mite *Oligonychus yothersi* McGregor (ICA 1989; Cárdenas 1983, 1985). These arthropods have not became serious pests due to the fact that these agroecosystems are quite stable with a great biodiversity, which favors the development of beneficial agents and maintain in equilibrium the potential pests present in the farms. On the other hand, in the coffee growing areas, insecticides were not used indiscriminately and it is recognized that Colombia is the only country in the world where the coffee plantations were handled without the use or with very low use of insecticides up to the arrival of *H. hampei* (Bustillo 1991). This situation of equilibrium has been affected with the presence of this pest.

Implementation of an Integrated Pest Management for the control of the Coffee Berry Borer in Colombia

Different strategies are needed to control the Coffee Berry Borer, such as: cultural practices, crop agronomical management, which can reduce insect populations, the protection of beneficial fauna, and the introduction of exotic natural enemies and entomopathogens. Among these are the parasitoids: *Cephalonomia stephanoderis* Betrem, *Prorops nasuta* Waterston, *Phymastichus coffea* La Salle and the fungus *Beauveria bassiana* (Báls.) Vuillemin (Bustillo 1991, 1995; Benavides *et al.* 1994; Orozco 1995; Orozco y Aristizábal 1996). These strategies are covered by the concept of Integrated Pest Management (IPM) (NCA 1969; Rabb y Guthrie 1970; Andrews and Quezada 1989). The IPM focuses in a series of principles and concepts on pest control which are integrated and in a theoretical way are proposed to establish an ecological guideline in the solution of a pest problem. So the IPM is flexible, dynamic and always susceptible of improvement, even though its comprehension and adoption by the farmers may be difficult. In the case of *H. hampei* the IPM program has been defined as: the use of a series of control measures (cultural, biological and chemical) to reduce coffee berry borer populations to levels which can not cause economical damage and which allows the farmers the production of coffee for exportation in a competitive way. The control measures used must be compatible and should not cause deleterious effects to the farmers living in the coffee zone, nor to the fauna, and do not contaminate the coffee ecosystem. (Bustillo *et al*. 1998).This concept is now extended to the Integrated Crop Management (ICM), which includes besides all the above mentioned tools, all the agronomical practices which are not directly pointed to the borer control, but if they are implemented can contribute indirectly to reduce borer populations (Bustillo 2002).

The implementation of an IPM program for the control of the Coffee Berry Borer in Colombia begins with sampling and determining an economic threshold level. The damage caused by Coffee Berry Borer creates the necessity to take efficient control measures, in the right moment when the insect menaces the coffee crop. Therefore, an important requirement in an IPM program is to measure in the field the insect population and correlate this population with the final damage to the crop. In the case of *H. hampei*, the sampling consists on taking, from a hectare (sampling universe), 30 trees randomly (sample size), selecting a productive branch containing 30 to 100 coffee berries (sampling unit) and then counting total number of berries in the branch and total number of berries infested by the coffee berry borer. The infestation level is the result of dividing the total number of infested berries over the total counted coffee berries (Bustillo *et al.* 1998, Decazy 1990b, 1990c, Baker 1989, Baker *et al.* 1989, Muñoz 1988). The sampling should be done in a monthly basis in each coffee plot to follow up the borer populations and deciding control measures timely (Bustillo *et al.* 1998). By going through the coffee plots, allows to the evaluator localize sites where there is a high concentration of insect borer population, and in these marked places the farmer should intensify the control measures. On the other hand, when evaluation takes place, a sample of 2 – 3 infested berries per tree should be taken, to determine the borer internal population and mortality, recording also the position, inside or outside the coffee berry (Bustillo *et al*. 1998). The level of infestation, the position of the borer inside the berry, and the location inside the plot, allows the farmer to take good insect control decisions (Bustillo *et al*. 1998, Bustillo 2002). The infestation levels cannot surpass 2% in field conditions during the critical period which is described as the moment when the coffee berries are most susceptible to the insects attack such as 120 days after flowering.

Table 1. Worldwide distribution of *Hypothenemus hampei*

Region	Country	Year reported	Reference
Africa	Gabon	1901	(Beille 1925)
	Chad	1902	(Sponagel 1994)
	Dem. Rep. Congo	1903	(Leplae 1928)
	Central African Republic	1904	(Breilid et al. 1997)
	Uganda	1908	(Gowdey 1910)
	Côte d'Ivoire	1922	(Beille 1925)
	Kenya	1928	(Abasa 1976)
Asia	Java	1909	(Hagedorn 1910)
	Sri Lanka	1910	(Vernalha et al. 1965)
	Sumatra	1919	(Corbett 1933)
	Malaysia	1929	(Corbett 1933)
	New Caledonia	1948	(Bugnicourt 1950)
	Philippines	1960	(Breilid et al. 1997)
	Fiji	1961	(Breilid et al. 1997)
	Tahiti	1963	(Johnston 1963)
	India	1990	(Sreedharan et al. 1994)
	Vietnam	1969	(Schmutterer, H. 1969)
America	Brazil	1922	(Bergamin 1946)
	Surinam	1960	(Bustillo et al. 1998)
	Peru	1962	(Amaral 1963)
	Guatemala	1971	(Hernandez and Sanchez 1978)
	Honduras	1977	(El Cafe de Nicaragua 1979)
	Bolivia	1978	(Romero 1990)
	Jamaica	1978	(Reid 1983)
	Mexico	1978	(El Cafe de Nicaragua 1978)
	Ecuador	1981	(Ruales 1997)
	El Salvador	1981	(Decazy 1987)
	Nicaragua	1988	(Monterrey 1991)
	Colombia	1988	(Bustillo 1990)
	Dominican Republic	1995	(Guharay and Monterrey 1997)
	Venezuela	1996	(Rosales et al. 1998)
	Costa Rica	2001	(Promecafe 2002)
	Puerto Rico	2007	http://uprm.edu
	United States	2010	http://hawaii.gov

The basis of the IPM to control *H. hampei* is Cultural Control (Benavides *et al*. 2002). It has been demonstrated that in coffee plantations after harvest, 10% of the coffee berry production remain on the trees and in the ground (Chamorro *et al*. 1995). If this population of berries is infested with the Coffee Berry Borer, then the insect can continue its reproduction. Cultural control consists then on timely harvesting the coffee berries before they drop onto the ground. If needed, coffee berries should be hand picked from the ground or using engine powered devices (Figure 1) (Bustillo 2002). The over mature berries and especially the dry ones, when infested by Coffee Berry Borer are the reservoir for borer populations that will infest the next coffee berry production. The dispersal of Coffee Berry Borer adults should be avoided (Benavides 2010), since 64 to 75% of the total population of Coffee Berry Borer individuals are taken to the processing area during harvest time (Moreno *et al*. 2001) and then fly back to the field (Castro *et al*. 1998). Studies in Colombia demonstrated that the timely harvest and collection of ripe berries left by the pickers, reduced levels of infestation from 70% to less than 6% during a coffee production cycle (Saldarriaga 1994, Peralta 1995). Later studies have shown that it is feasible to improve the efficacy of harvesters by allowing them to leave on the trees less than five ripe coffee berries, after a harvest pass (Díaz y Marín 1999). This has been also proved under a farmer's participatory research approach (Aristizábal *et al.* 2002, 2004a).

Figure 1. Vacuum machine prototype to collect coffee berries from the ground.

There are several tasks which need to be implemented in the farms to contribute to Coffee Berry Borer population reduction (Bustillo 2002): (1) planting resistant varieties such as Castillo®, which is resistant to coffee leaf rust (Alvarado and Moreno 1999), then does not require the use of fungicides and then allows the use of entompathogens to biologically control *H. hampei,* besides its coffee berries are more heavily attached to the tree and they do not fall as easily onto the ground; (2) planting the coffee trees in the field using larger distances among trees in array of 2 x 1 m and leaving two stems per tree (Mestre y Salazar 1995) allow workers to move around the coffee fields more efficiently to perform different tasks such as harvest, evaluation of infestation, sprays to control the borer, among others; (3) cutting down old trees in a planned coffee renovation is recommended after the fifth harvesting year (Mestre y Ospina 1994a, 1994b), this will ensure a proper picking procedure and would allow the implementation of cultural control practices; (4) The use of a weed selector (Figure 2) (Rivera 1997, 2000) in order to control unwanted weeds in the coffee plantations and leaving those that do not compete with the coffee plant, are good nectar producers and feed natural enemies of coffee insect-pests (Salazar and Baker 2002); and (5) avoiding the return to the coffee fields of flying Coffee Berry borers by

means of using traps (Aristizábal *et al.* 2002), depulping the coffee without water during the coffee processing (Alvarez 1991) and using devices to dry coffee beans using solar or mechanical energy (Benavides 2010b).

Figure 2. Weed selector to maintain green covertures on the coffee soil to avoid erosion and favor biodiversity of beneficial fauna.

Besides Cultural control and agronomical practices, there is need to complement the IPM program with biological natural agents to control the Coffee Berry Borer, as well as the use of chemical insecticides in a safe and timely manner. Thus, Biological control plays an important role in this program, by means of using native beneficial fauna, exotic imported parasitoids and entomopathogenic fungi and nematodes. Native predators, parasitoids, nematodes and entomopathogenic fungi have been described in Colombia (Bustillo 1995, Vera *et al.* 2007, Lara *et al*. 2004). These findings confirmed the importance of preserving the Colombian coffee ecosystem with control measures that would not affect the beneficial fauna, so the farmers have to spend less effort in the control of this insect. Ants are playing an important role in the biological control of *H. hampei*. Vélez (2002) found *Solenopsis geminata*, *Dorymyrmex* sp., *Pheidole* sp., *Mycocepurus smithii* and *Gnamptogenys* sp. (Figure 3) being capable of preying borer adults while attacking coffee berries. Armbrecht and Gallego (2007) also experimentally tested high predation levels of ants on *H. hampei* inhabiting berries on the soil.

Figura 3. Ant of the genus *Gnamptogenys* preying on coffee berry borer adults boring into coffee berries (Photo G. Hoyos).

Other Biological control strategy is the introduction of natural enemies not present in Colombia. Three parasitoid species were introduced from Africa via quarantine in England: *Cephalonomia stephanoderis* Betrem, *Prorops nasuta* Waterston y *Phymastichus coffea* La Salle. Massive production systems of these species have been documented (Orozco 1995, Portilla y Bustillo 1995, Orozco y Aristizábal 1996, Bustillo *et al*. 1996, Orozco 2002). The methodologies of these processes were made available to 11 private laboratories and the production of about 2000 millions was contracted by the Coffee Growers Federation during a period of five years (1995 – 1999). About 1700 millions of these parasitoid species were released in coffee infested fields throughout the country, with the initial purpose of establishing them (Bustillo *et al.* 1998). Field studies have shown the potential of *C. stephanoderis* and *P. nasuta* to reduce infestation levels of coffee berry borer (Salazar y Baker 2002; Bacca 1999; Benavides *et al*. 1994; Aristizábal *et al.* 1997). A similar program was conducted with *Phymastichus coffea*, adult parasitoid of *H. hampei.* A massive production system was developed (Orozco and Aristizábal 1996, Orozco 2002) and after testing its selectivity to other Scolytinae species (López – Vaamonde *et al.* 1997), field releases were authorized in Colombian coffee plantations. *P. coffea* parasitize the *H. hampei* adult that is entering the coffee berry (Figure 4), being an ideal complement to the other two species. Under field conditions this parasitoid has a high searching capacity for *H. hampei* populations (Vergara *et al*. 2001a, Echeverry 1999), even at low population levels (< 5% infestation) (Vergara *et al.* 2001b); and greater parasitism when the borer is penetrating berries of 70 to 170 days of development (Jaramillo *et al.* 2002, 2005). Aristizábal *et al*. (2004b) showed the importance of these parasitoids in the regulation of Coffee Berry Borer populations. However, samples taken from releasing sites three years later did not confirm establishment of this species. Recent studies have shown only the field recovery of *P. nasuta*, in coffee plantations in Colombia (Maldonado and Benavides 2007). Apparently, this species is best adapted to conditions in the Neotropic. Even though mass produce the Coffee Berry Borer parasitoids is expensive, current efforts are made to rearing *H. hampei* on artificial diets (Ruíz *et al*. 1996, Portilla and Bustillo 1995, Portilla and Streett, 2006) then producing the parasitoids in a more cost effective procedure.

Figure 4. *Phymastichus coffea* adult parasitizing an adult of *H. hampei* entering the coffee berry (Photo G. Hoyos).

Insect nematodes are considered a good alternative to decrease the population of Coffee Berry Borer that is present in infested berries onto the ground. In Colombia, it has not been recorded any natural infection by entomonematodes (Bustillo *et al.* 2002). However, the literature indicates records of *Panagrolaimus sp*. (Panagrolaimidae) and *Metaparasitylenchus hypothenemi* Poinar (Allantonematidae) nematodes infecting borer populations naturally in coffee plantations in India and Mexico (Varaprasad *et al*. 1994, Castillo *et al.* 2002). Research on the nematodes *Steinernema colombiense* López and *Heterorhabditis bacteriophora* Poinar, found in the soil of Colombian coffee ecosystems (López *et al.* 2008) have focused to determine the pathogenicity on the coffee berry borer, its behavior and strategy of host finding (Molina and López 2002; 2003), life cycle (López 2002), evaluation of application systems (Lara and López 2005) and evaluations under greenhouse and field conditions in small scale (Giraldo, 2003; Lara *et al*, 2004). Other studies cover evolutive relationship and species diversity of nematodes in Colombia (López *et al.* 2007). The species *S. colombiense* and *H. bacteriophora* are able to find and infect coffee berries infested by *H. hampei* (Lara *et al*. 2004). In Colombia, in spite of development of massive techniques in some countries as Germany and United States to produce entomonematodes, they are only produced in small scale using live insects such as *Galleria mellonella*, which made the process too expensive. This is a limitation if the market demands these organisms.

The cosmopolitan entomopathogenic fungus *Beauveria bassiana* is found naturally infecting the Coffee Berry Borer (Posada and Bustillo 1994). It has been sprayed in field conditions after artisan (Antía *et al.* 1992, Marín and Bustillo 2002) and industrial production (Morales *et al.* 1991). The Coffee Growers Federation in Colombia financed a large program to disseminate *B. bassiana* in all regions where the insect was dispersing (Bustillo 2002). The development of bioassays (González *et al*. 1993, Posada *et al.* 2002) to select virulent isolates, the instructions to reactivate the fungus in insects (Bustillo and Marín 2002) and the protocols for quality control of fungus produced using artisan and industrial processes (Vélez *et al*. 1997), have allowed a better control and improvement on the commercially available fungi formulations. Several studies on the efficacy of *B. bassiana* under field coffee conditions have been carried out (Bustillo *et al.* 1991, 1995, Bustillo and Posada 1996, Flórez *et al*. 1997). Results are variable and influenced by the quality and concentration of the fungus, climatic and crop management conditions. Levels of control may fluctuate from very low values near to 20% to high levels of 75%.

Current researches are now directed to improve the efficacy of these fungi to control the coffee berry borer. Studies have been conducted on selection, characterization of isolates of *B. bassiana* and *M. anisopliae*, having in consideration their morphology (Padilla *et al.* 2000), pathogenicity

(Jiménez 1992, Bernal *et al.* 1994), physiological characteristics and reproduction (Valdés *et al.* 1999, Vélez *et al*. 1999, 2001) and using molecular techniques (Valderrama *et al.* 2000, Gaitán *et al.* 2002). Recently, there is interest in the genetic transformation of these fungi with genes that could increase its virulence and be more efficient under field conditions to control the Coffee Berry Borer (Góngora *et al.* 2000, Góngora 2005, Rodríguez and Góngora 2005), but there are not yet regulations in Colombia to manipulate transgenic microorganisms, which delays the advances in this area. On the other hand there are evidences of more efficient entomopathogens under field conditions by spraying mixtures of different strains to control *H. hampei* (Cárdenas *et al.* 2007).

The use of insecticides to control the coffee berry borer should be carried out only when technically needed, that is when levels of borer infestation surpasses 2% during the critical period of the attack of the Coffee Berry Borer, and at the moment that more than 50% of the flying females are still outside the coffee berries. These two parameters are obtained with the proposed sampling above mentioned (Bustillo 2002). After testing more than 50 chemical insecticides, three less toxic (category III) molecules were recommended for achieving similar efficacies than endosulfan: fenitrotion, phenthoate and chlorpyrifos (Villalba *et al.* 1995). They should be applied in a localized area where the insect is present and using the appropriate spray technology to achieve a good borer control (Villalba *et al.* 1995, Bustillo *et al.* 1998, Posada *et al.* 2004). In field conditions the control of *H. hampei* with chemical insecticides is very erratic. To explain failures different factors need to be taken in consideration such as correct dosage, calibration of equipment and operators, field topography, environmental conditions at the moment of spraying, and the proper moment to apply the insecticide according to the borer attack.

Impact of our research on *H. hampei* in the Colombian coffee industry is supported by the statistics of Almacafé, the coffee organization in charge of coffee storage and exportation (Figure 5). Levels of infested green coffee by this insect in Almacafé have been reduced greatly. In 1994 infestation average levels were about 16% of all stored green coffee, in 2002 were below 4.1% (Abisambra 2004) and in 2007 dropped to 2.1% (FNC 2007). The adoption of coffee management strategies to reduce populations of *H. hampei,* have contributed to the commercialization of the Colombian coffee without any obstacle and favored the coffee economy which at current prices could represent savings around US$120 million dollars annually. The social impact can be summarized as the preservation of the environment for using less toxic insecticides and no toxic bioinsecticides, reducing costs and maintaining high coffee quality in the market.

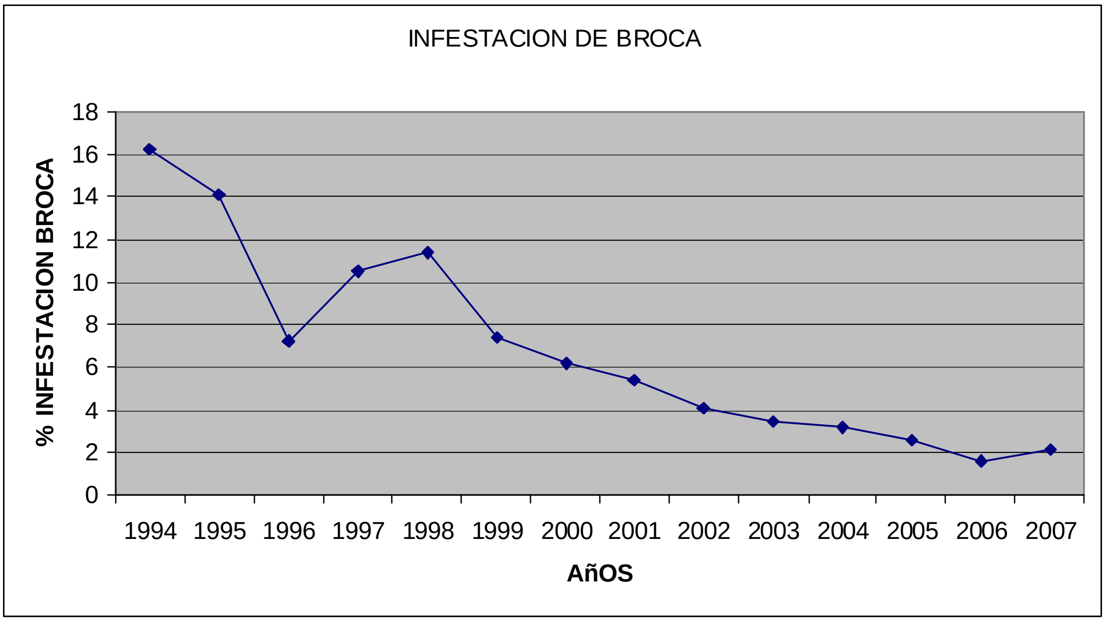

Figure 5. Records of percentage of dry parchment coffee infested with *H. hampei*, stored in Almacafé, Colombia from 1994 o 2007. (Reports of Almacafé).

Unfortunately, irrational use of insecticides has caused several problems, such as insect resistance (Brun *et al.* 1989). This situation is accentuated by the sib-mating behavior and the functional haplodiploidy exposed by the Coffee Berry Borer (Benavides *et al.* 2005), which allows the fixation of mutations in few generations of this matrilineal species. This is the case of resistant of *H. hampei* to endosulfan reported initially in New Caledonia (Brun *et al.* 1989), tested later through molecular studies (Ffrench-Constant *et al*. 1994) and found that depends on the gene *Rdl* which codifies a subunit of the receptor of acid γ-aminobutiric (neurotransmitter GABA), that is responsible to activate the chloro channel during synapses. This gene of resistance was favored in New Caledonia through selection processes. Insecticides belonging to the group of cyclodienes as DDT, lindane and endosulfan, were continuously applied in a generalized manner since 1966 and in less than 20 years, levels of Coffee Berry Borer infestation reached the historic maximum and the resistance was documented (Brun *et al.* 1989). This resistance has also been described in Colombia (Góngora *et al.* 2001, Navarro *et al.* 2010). The appearance of this mutation in *H. hampei* population in Colombia followed not appropriate control practices performed by few non-adopting IPM coffee farmers that sprayed irrationally endosulfan for several years, thus the frequency of this gene in the borer population increased and chemical insecticide to control Coffee Berry Borer failed. Initially, in order to confirm the presence of the Dieldrin resistance allele (Rdl^R) gene in Colombian Coffee Berry Borer populations, concentration-mortality responses for individual coffee berry borer lines reared from four different Colombian coffee areas were estimated using a Potter Spray Tower (Burkard Manufacturing Co). Three different concentrations of endosulfan were tested on insects reared from coffee areas where endosulfan resistant was suspected: low dosage of 400 ppm, medium dosage of 10,000 ppm and high dosage of 20,000 ppm. Susceptible Putative (SS) insects were discriminated successfully from heterozygous putative (RS) and homozigous (RR) insects with the low and high dose. The low dosage caused a 100% mortality rate in the (SS) susceptible strains after 24 hours endosulfan exposure; all (RS) and (RR) survived. The survivors were then sprayed with either 10,000 or 20,000 ppm and evaluated after 6hrs. The 20,000 ppm dosage caused 79.3, 74.02, and 94.64% mortality in the (RS) strains from three studied sites. All homocigotes (RR) lines used as control survived the high dosage of 20,000 ppm. The individuals were then genotyped and the genetic

condition was corroborated by PASA. The results obtained confirmed the presence of the gen *Rdl* in Colombian populations of *H. hampei*. PASA showed to be an appropriate technique to identify resistant populations from the field, based on this, a melting temperature (T_m) shift genotyping method that relies on allele-specific PCR was described for insecticide resistance-associated single nucleotide polymorphism (SNP) at the *H. hampei Rdl* gene (Navarro *et al.* 2010). Later findings on the resistant populations have shown biological disadvantages associated to the borer individuals. Homozygous resistant lines showed a marked decrease on progeny production while compared to homozygous susceptible individuals, as well as a longer survivorship time of the resistant borers (Figure 6 and 7).

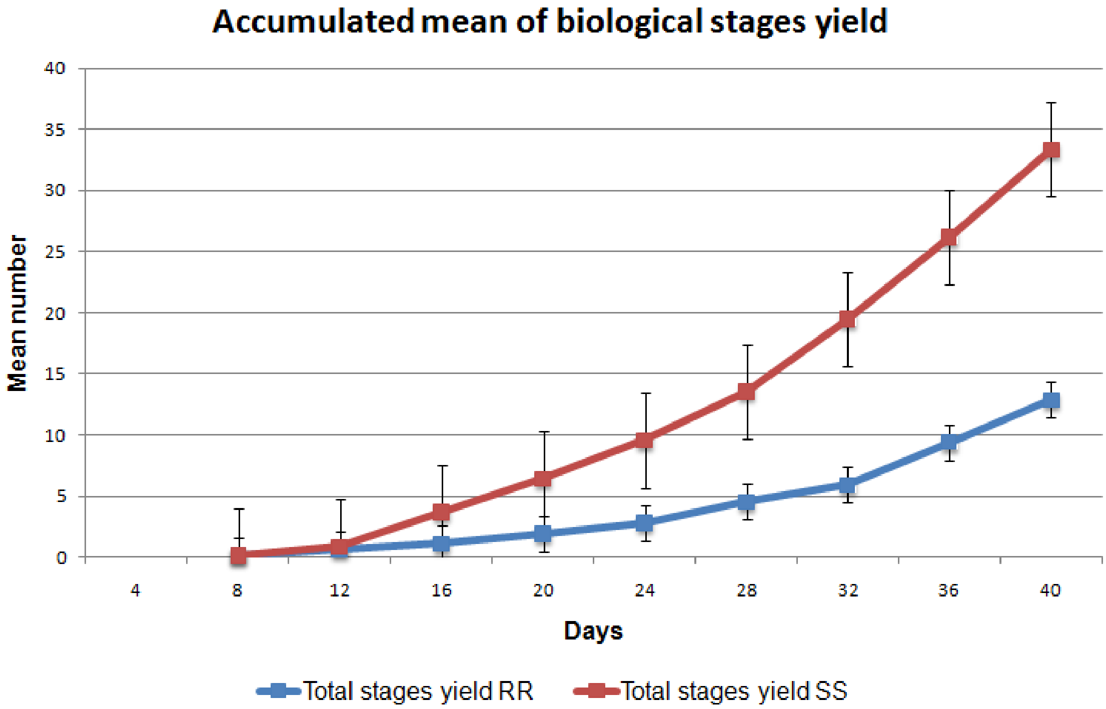

Figure 6. Total progeny produced by Coffee Berry homozygous resistant RR and susceptible SS to endosulfan insecticide in lab conditions.

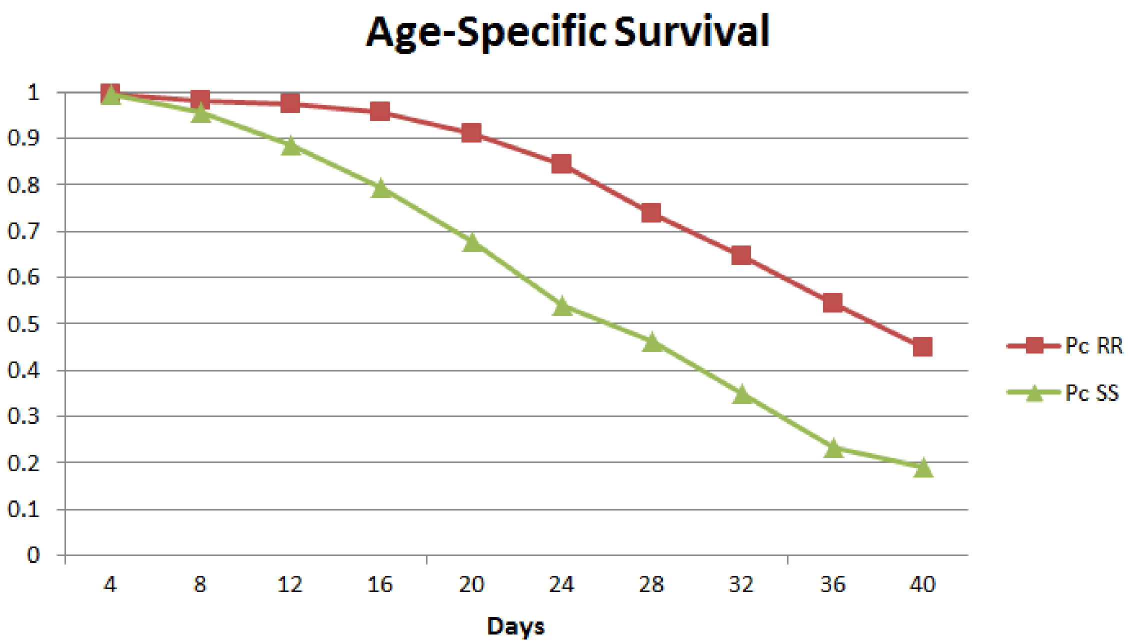

Figure 7. Survivorship of adult Coffee Berry homozygous resistant Pc RR and susceptible Pc SS to endosulfan insecticide in lab conditions.

Right now it is considered that in view of the market restrictions on insecticide residues in exporting commodities and with the emphasis on specialty coffees, environmentally friendly strategies such as *B. bassiana* are now considered as a very valuable alternative in the reduction of *H. hampei* (Bustillo 2004).

***Beauveria bassiana* as a strategy to overcome insecticide resistance**

The emergence of chemically resistant insects as well as the desire to decrease reliance upon chemical pesticides in favor of more eco-friendly and organic production compatible methods has lead in Colombia to the investigation of alternate biologically based control strategies, one of which is the use of entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Vuillemin, which is actively being examined as a biological agent to control the Coffee Berry Borer.

Indeed, in the Colombian coffee ecosystem, this fungus is a natural controller of the *H. hampei* (Bustillo 2006). Furthermore, there are no known diseases of *H. hampei* caused by either bacteria or viruses. High Coffee Berry Borer mortality caused by the fungus *B. bassiana* has been reported on Indian coffee plantations (Haraprasad et al 2001) and Mexico (De La Rosa et al 2000) as well.

B. bassiana is a broad host range insect pathogen that has been EPA approved for use as an insect pest biological control agent and is available from various commercial companies world-wide (Goettel *et al*., 2005) including Colombia.
Since the arrival of the Coffee Berry Borer to Colombia, *B. bassiana* has been related to the insect and it was reported attacking insects since 1990 (Velez and Benavides, 1990). Today, the fungus is considered a natural controller of the pest because it is found infecting the insect in all the countries where Coffee Berry Borer has arrived.

However in order to get to this levels, when the insect arrived, the research developed in Cenicafé, allows to produce enough fungi, to spray the coffee-invaded area, free of cost to the farmers by the Coffee Growers Federation during three years. This program allowed the Coffee Berry Borer to be exposed to the fungus causing infections on its population. This is a good example of classical biological control through the introduction of a beneficial organism not present in the insect population. Between 1992 and 1995, 200 tons of fungi were produced ("Strain Cenicafé") by the Colombian Coffee Growers Federation and private laboratories. Between 1996 and 1998 this production was raised to 400 tons to control the borer. The developments of this research allowed the formation of several private laboratories in Colombia dedicated to the production of several species of entomopathogenic fungi not only to control coffee pests but several other insect pests of different crops. Biological commercial laboratories from Colombia have founded new laboratories in other Latin American countries such as: Ecuador, Brazil, Peru, Panama, Costa Rica and Guatemala.

Today the natural control caused by the fungus in the Colombian coffee zone has been calculated around 10%. If *B. bassiana* has not been present, the economic losses in the Colombian coffee industry will be much higher (Góngora *et al.*, 2009). Today *B. bassiana* is part of the Coffee Berry Borer - IPM strategy and its application is recommended by Cenicafe (Bustillo 2002).

However the use of high concentrations of *B. basassina* spores is costly and one of the ways of reducing the biological control cost is to increase the virulence of the strains and its resistance to adverse environmental conditions. This will allow the reduction of the spore doses required for controlling the insect and to diminish the mortality time, in such a way that the insect will cause a minor damage in the berries, helping to reduce the problem of the delaying in mortality caused by the fungus comparing to the chemical insecticides (St Leger and Wang 2010).

Historically fungal pathogens of plant and insect pests have not met expectations because of slow kill, failure to identify strains active at low doses and inconsistent results compared with the chemicals they compete with (Gressel 2007, Gressel *et al*., 2007). These failures may be exasperated by our incomplete understanding of the biological and genetic factors that make fungi effective. However, lack of efficacy could also be inbuilt because an evolutionary balance may have developed between microorganisms and their hosts so that quick kill, even at high doses, is not adaptive for the pathogen (St. Leger and Wang, 2010).

The combination of geographical location and agricultural practices in their plantations throughout Colombia also, results in a wide variety of microclimate conditions that can affect the performance of a biological control agent (Cruz et al 2006). Coffee cultivation conditions in the country range in altitude from 1,000 to 1,800 m above sea level, with precipitations during the rainy seasons and solar intensities that change widely among locations and along the year (Cenicafe 2010). Similarly, farmers use plant densities that vary between 5,000 to 10,000 plants per hectare, with or without shade trees. This affects the consistency of biological control performance when compared to spraying synthetic insecticides under homogeneous cultivation conditions, decreasing the appeal for biological product applications by the farmer. Therefore, a constant improvement in biocontrol technology is required as the need to reduce economical and environmental costs of control measures plays a critical role in an agricultural industry such as coffee production.

As part of this goal "constant improvement in biocontrol technology**"** Cenicafé has collected throughout the years 196 isolates of *B. bassiana* from diverse hosts and geographic origins (Cruz et al 2006. Góngora et al 2009). Even though the worldwide population of this species has been found to have a low genetic diversity (St. Leger *et al*. 1992, Glare and Inwood 1998, Castrillo *et al*. 1998, Coates *et al*. 2002, Gaitan *et*

al. 2002), *Beauveria*'s isolates show differences in their virulence (Bustillo and Posada 1996, Velez *et al.* 1999, Cruz 2006). Nevertheless, until now, only the isolate Bb 9205 has been the only genotype distributed for pest control purposes in Colombian coffee plantations.

We know that the current practice of production and application of clonal isolates selected because of their virulence towards an insect can result in a short and limited suppression of the pest (Boucias *et al*. 2000). Tigano-Milani (1995) proposed the hypothesis that more than one haplotype (clone) may be required to initiate and maintain an epizootic in a natural and heterogeneus insect population, such as the one found under Colombian conditions. The variability of the strains is the factor that would allow the fungus to adapt to changing environmental conditions and to successfully attack different insect populations.

Experimentally, however, the role of strain diversity may be hard to establish due to the difficulty to identify intraspecific variations using classical morphological and biochemical methods, and therefore making laborious the monitoring and tracking of multiple strains in the same infection. In this sense, DNA profiles have been used as powerful and sensitive tools to precisely identify individual strains infecting a host population (Wang *et al*. 2004), but to date only two assays using molecular markers on strain mixtures or coformulated strains of entomopatogenic fungi have been reported. Leal-Bertioli *et al.* (2000) distinguished two co-formulated strains of *Metarhizium* infecting *Phaedon cochleariae*, while Wang *et al*. (2004) differentiated two strains of *Beauveria* infecting *Galleria mellonella*. In both *in vitro* assays, one strain dominated over the other, and parasexual recombination or heterokaryon formation was detected. In nature, however, the presence of diverse strains of *B. bassiana* in samples collected from the same pest outbreak has been reported, suggesting that successful epizootic development requires the involvement of genetically distinct genotypes (Castrillo *et al*. 1998). Besides, under field conditions, monitoring of massive applications of two *B. bassiana* strains resulted in co-infection or genetic recombination of the isolates (Wang *et al*. 2004).

Base on those hypothesis we select ten *B. bassiana* strains, previously characterized by RAPDs (Gaitan *et al.* 2002). They were reactivated from Cenicafe's collection of entomopathogens. Six of these isolates came from various places in Colombia (Bb 9001, Bb 9005, Bb 9010, Bb 9011, Bb 9119, Bb 9205), and the rest came from Thailand (Bb 9016), Philippines (Bb 9020 and Bb 9023) and Canada (Bb 9024). Genomic DNA from the strains was characterized using ITSs and β-tubulin sequences as well as AFLPs markers (Cruz *et al*.2006).

For the ITS: A PCR fragment containing the 3´end of the 18S ribosomal RNA gene, the complete ITS1, 5.8S and ITS2, and the 5´end of the 26S, was amplified using the primers ITS 1 and ITS 4 described by White *et al*. (1990. An ITS amplification product of around 569pb was obtained for all the isolates. The ITS sequences for each on of strains were deposited in the GenBank. For the β-tubulin sequences, a PCR reactions with the primers Bt-T2m-Up and Bt-LEV-Lo1, described by de Jong *et al*. (2001), were used to amplify the 3' end intron of the β-tubulin gene of *Beauveria* sp. and part of its flanking exons. All the isolates displayed a 982bp PCR amplification product, corresponding to the 3' intron and parts of the flanking exons

A high number of informative and reliable sets of AFLPs for each one of the isolates was obtained. Only consistent bands with molecular weight between 200-500bp were scored to generate a binary matrix with 120 markers, and a PCOORDA analysis was done.
Based on the grouping analysis obtained with ITSs and β-tubulin, the isolates were clustered in three genetic groups. Group 1 made up by isolates: Bb 9001, Bb 9005, Bb 9010 and Bb 9020; Group 2 formed by isolates: Bb 9011, Bb 9016 Bb 9119 and Bb 9205, and Group 3 composed by

isolates: Bb 9023 and Bb 9024. The cluster analysis also confirmed the low but significant intraspecific genetic diversity present among the strains.

Single strain virulence towards the coffee berry borer under laboratory conditions were done, virulence tests were carried out according to the method described by Gonzalez *et al.* (1993). *H. hampei* adults were obtained from a laboratory colony maintained in parchment coffee (Bustillo *et al*. 1998). For each treatment, insects (15 individuals per treatment with 4 replicates) were inoculated by dipping them into a 10 ml spore suspension of $1x10^6$ spores ml^{-1}. They were then transferred to individual containers with a filter paper at 25 °C and 80% humidity. Insect mortality was recorded at 24 hours intervals during 8 days, discriminating between death by fungal infection, with the observation of mycelium on the cadaver, and death by other causes. The virulence ranged between 90% and 57.5%

All the inoculations with mixtures resulted in coinfection events. Combinations of genetically similar strains showed no significant differences when their virulences were compared. However, mixtures of genetically different strains led to both antagonism and synergism. The lowest virulence percentage (57%) was obtained by putting together the most virulent strain of each group (Bb 9020, Bb 9023, Bb 9205), contrary to the highest virulence percentage (93%) that resulted from **mixing the three least virulent strains (**Bb 9001, Bb 9119, Bb 9024) .

Based on those first results Cardenas *et al*., 2007, evaluated again the virulence of all the individual strains and mixtures, the virulence assay results under lab conditions are showed in Table 2.

Table 2. Coffee Berry Borer mortality percentage caused by *B. bassiana* ($1x10^6$ spores /ml) in lab conditions.

Treatments	***H. hampei* Mortality caused by B. bassiana**	
	Average(%)	**C. V.(%)**
Bb 9020	81.67 bcd	4.99
Bb 9023	83.33 bc	6.19
Bb 9205	88.33 b	4.62
Bb 9001	76.67 cd	6.73
Bb 9119	73.33 de	7.04
Bb 9024	53.33 f	9.68
Mixture A- **most virulent strain** (Bb 9020, Bb 9023, Bb 9205)	65.00 e	8.43
Mixture B- **least virulent strains** (Bb 9001, Bb 9119, Bb 9024)	100.00 a	0.00
Commercial formulation	83.33 bc	6.19

Averages with no common letters indicate differences among treatments according to Tukey (P=0.05) comparison test.
C.V. Coefficient of variation

With the same strains and mixtures we evaluated the virulence under field conditions. For this, in the coffee farm “Tamboral”, located in Manizales - Caldas, in a 20 month coffee plot Colombia variety , twenty-five-tree plots with three repetitions distributed through a completely randomized design were used. One coffee tree per plot and a branch with 50 coffee berries were selected to

make artificial infestations of the insect. After 24h, the infested branches were sprayed using a dose of 2x107spores/branch for each treatment. After 30 days, the insects mortality was assessed through berries dissection.

In the coffee plantation, the highest mortality was registered with the low-virulence strain mixture (66.6%) witch it was higher that the mortality caused by individual strains or other mixture evaluated. So far a mortality of 66.6% is the highest observed due to Beauveria sp under field conditions in Colombia (Table 3).

Table 3. Coffee Berry Borer mortality percentage caused by *B. bassiana* ($1x10^{6}$ spores /branch) in field conditions.

Treatments	**Mortality(%)**	
	Average	**C. V.(%)**
Bb 9020	53.1 b	8.7
Bb 9023	55.5 ab	16.2
Bb 9205	59.6 ab	11.4
Bb 9001	54.1 ab	15.5
Bb 9119	58.3 ab	16.4
Bb 9024	55.1 ab	21.9
Mixture A- **most virulent strain** (Bb 9020, Bb 9023, Bb 9205)	60.2 ab	11.5
Mixture B- **least virulent strains** (Bb 9001, Bb 9119, Bb 9024)	66.6 a	15.8
Commercial formulation	56.6 ab	17.7
Testigo dentro de la parcela	19.5	63.9
Testigo fuera de la parcela	8.4	63.7

Averages with no common letters indicate differences among treatments according to Tukey (P=0.05) comparison test.
C.V. Coefficient of variation

The results indicated the promising potential of designing strain mixtures as an alternative for the biocontrol of *H. hampei* and other pests, and provides tools for the understanding of the ecological dynamics of entomopathogen populations under natural conditions.

In addition, the problem of the Coffee Berry Borer is not only limited to the insect population that attacks the coffee berries from the tree branches but also exist a permanent insect population that remain in the berries that have fallen onto the ground, which are at the base of the trees after coffee harvesting and act as a driving source for new infestations (Castaño *et al*. 2005, Bustillo 2002, Benavides 2010a). The fallen berries are reservoirs for adult insects and are food for larvae. When conditions are appropriate, i.e. under high humidity and temperature conditions, adult insects that remain in the fallen berries fly to new coffee berries that are on the branches of the trees or that have fallen to the soil, penetrating growing or ripe berries and depositing eggs. The eggs hatch, and the larvae consume the seeds, damaging them and causing these berries to fall to the base of the tree. The larvae become adults, and those adults mate with the siblings and fly, repeating the entire cycle. Therefore, determining the efficacy of *B. bassiana* on Coffee Berry Borer populations that emerge from fallen infested berries and infest the berries in the trees will contribute to improving pest control strategies and decrease the losses caused by this pest.

Base on this, we evaluated the effect of the application of the *B. bassiana* to infested berries from the soil and its effect on the percentage of new berry infestations from the trees. The experiment was done in 2 different Colombian coffee experimental stations during 2009: Paraguaicito-Quindío and Naranjal-Caldas. The research at Paraguaicito was conducted between February and March. Paraguaicito is located at altitude of 1210 m above sea level, has during those months on average 23°C, 77% RH, and sandy-loam soils. The coffee trees were 3 years old in second harvest, with average size of 1.7 m, and they were planted at a distance of 1.30 m × 1.30 m. Research at Farm 3was conducted between July and August. Farm 3is located at altitude of 1381 m, has during that period of the year on average 21.4 °C and 68% RH, and clay-loam soils. The coffee trees were planted at a distance of 2.0 m × 1.0 m, and they were 2 years old after stump in second harvest. At both locations, *Coffea arabica* variety Castillo trees was used and they had berries of 120 to 150 days old. The plots had a slope of no greater than 20%.

The treatments consisted of application of *B. bassiana* strain Bb9205, a mixture of three *B. bassiana* strains Bb9001, Bb9119, and Bb9024, a commercial formulation, and a control (water) to infested berries placed at the base of a coffee tree. The experimental plot in each location was formed by 9 coffee trees in square with a 3 × 3 array of, in which the central tree was the sample unit. Each treatment was replicated 10 times and forty experimental plots were established.

In the sample unit trees, all infested coffee berries on the tree branches and the fallen berries from the bases were removed. Then, 50 artificially infested coffee berries were placed on the ground next to the base of each tree. The coffee berries were infested with 4 adult females, 30 days previously to the set up of the experiment. The treatments were sprayed over the berries left on the ground one day later, the trees, and their bases were completely covered with net entomological cages. The four treatments were assigned according to a completely randomized experimental design.

After 30 days of establishing the treatments, the total number of berries per tree, the total number of infested berries, and the percentage of infestation per tree were recorded. Then, 50 infested berries were randomly collected from all branches on each tree and were dissected in the laboratory to register the position of the insects in the berries and the degree of penetration inside the berry. Positions A and B referred to Coffee Berry Borer individuals initiating the attack of the fruit, whereas positions C and D indicated that the insects were inside the seed (Bustillo 2006). Number of live Coffee Berry Borers, dead Coffee Berry Borers without the presence of fungus, and dead Coffee Berry Borers with signs of fungus were recorded. The dead Coffee Berry Borers with no fungus signs were placed in a moist chamber to add them into the insects killed by the fungus, if stated.

The results showed reduction on infestation levels ranged from 15 to 55% at Farm 3in all treatments with respect to the control. In Paraguaicito, there were differences in percentage of infestation between the mixture and the control, and the reduction was 38%. At Narajal the infestation decreased by 50% (2.2 fold) with treatment with the mixture of Cenicafé strains compared to the control, whereas the decrease in the percentage of infestation at Paraguaicito with the same treatment was 30% (1.6 fold) compared to the control treatment. At both locations, treatment with the mixture of Cenicafé strains had a greater effect on Coffee Berry Borer that emerged from the berries left on the ground, which caused a significant decrease in the percentage of infestation of berries in the tree.

In the berries dissected from treated tree, insect mortality was about 40% at both locations compared to 15% in the control and it also decreased the insect population inside the newly infested berries on the trees by 55 to 75% (Table 4). In general, we can conclude that the

application of the fungus on infested berries left on the soil can decrease the number of individuals of subsequent generations of Coffee Berry Borers (F1) by up to 55% at Paraguaicito. At Naranjal, the decrease in the number of eggs reached 90% after treatment, and the larvae decreased up to 87% compared to the control. Overall, the entire population was reduced at least 75% after fungal treatment, with the mixture of Cenicafé having the greatest effect. Previously, Aristizábal (2005) stated that treatment of berries on the soil with *B. bassiana* affected the Coffee Berry Borer in such a way that reduced the progeny of biological stages of Coffee Berry Borers produced by these infected insects in tree berries, but until now, the quantification of this reduction had not been examined.

Table 4. Mean numbers of biological stages of the Coffee Berry Borer present in berries collected from the field. Effect of *B. bassiana* application on Coffee Berry Borer emerging from berries left in the field and on the Coffee Berry Borer offspring.

	Station Paraguaicito–Quindio.			**Station Naranjal-Caldas**		
Treatment	**Eggs**	**Pupae**	**Larvae**	**Eggs**	**Pupae**	**Larvae**
Brocaril®	31.8* ± 10.72	0.0	37.7 ± 13.99	16.1* ± 6.22	0.4 ± 0.9	23.6* ± 10.11
Bb9205	58.3 ± 22.37	0.0	63.7 ± 31.05	29.9* ± 18.23	0.0	35.8* ± 16.7
Mixture	39.9 ± 21.85	0.4 ± 0.9	30.1 ± 20.87	14.6* ± 5.1	0.0	19.1* ± 12.17
Control	69.6 ± 25.93	0.0	63.2 ± 39.12	139.2 ± 27.25	0.0	149.1 ± 19.17

*** Average statistical differences compared with the control according to Dunnett's test (P = 0.05) per biological state.**

It has been reported that insects infected with an entomopathogenic fungus may alter their behavior during mating and oviposition (Goettel *et al.* 2005) decreasing their progeny. In the case of Coffee Berry Borer, the infection can cause physiological damage to insects in such a way that they cannot mate inside berries, or eggs do not develop after mating. Fungal infection can also induce aberrant behavior that can decrease the fitness of the insect. These behaviors include male copulating more with infected females, or infected females not copulating, which are behaviors previously reported in other species of insects (Watson and Petersen 1993; Roy et al 2006). The effect of strain mixture have been evaluated recently for other authors, mixture *Pseudomonas fluorescens* strains and *Enterobacter cloacae* have been used to boost biocontrol efficacy and consistency of potato maladies – dry rot, late blight, pink rot, and sprouting (Slininger et al 2010), In nature, the presence of diverse strains of *B. bassiana* in samples collected from the same pest outbreak has been observed, suggesting that successful epizootic development requires the involvement of genetically distinct genotypes (Castrillo *et al.* 1998). Besides, under field conditions, monitoring of massive applications of two *B. bassiana* strains resulted in co-infection or genetic recombination of the isolates (Wang *et al.* 2004).

We concluded that *B. bassiana* significantly decreased Coffee Berry Borer populations that emerged from fallen, infested, coffee berries and reduced future insect generations and the mixture was the most effective for decreasing the insect populations.

CONCLUSIONS

The coffee berry borer is a serious insect pest of coffee crops worldwide. Historically, the strategies to overcome this pest have not been aligned with environmentally friendly schemes and problems such us resistant to chemical insecticides arrived soon as expected.

An integrated pest management program in Colombia has proved to maintain the Coffee Berry Borer under the economic threshold, however more progress on less labor intensive strategies are needed.

Biological control agents have been introduced, conserved and augmented in order to naturally control Coffee Berry Borer. None, except the use of highly pathogenic fungi such as Beauveria bassiana, has proved to be economically and biologically effective to control Coffee Berry Borer in the field. Our results helped in the understanding of Insect –entomopathogen interaction and the development of a mixture of strains that could be very important for insect control not only in coffee but also in other crop.

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